that this rate is proportional to available substrate in the degradation pathway:

$$dS/dt = k(fT - S) = k(S_0 - S) \quad (4)$$

The solution of this equation is

$$S(t) = S_0[1 - e^{-k(t-t_0)}] \quad (5)$$

Since $T = S_0 + P_0 = S(t) + P(t)$, both sides of eq 5 can be multiplied by $100/T$ to yield

$$D(t)_{model} = D_0[1 - e^{-k(t-t_0)}] \quad (6)$$

This expression is formally identical with eq 2 which was fit to data from the pulse–chase experiment. The reciprocal of the rate constant (k^{-1}) defines a time scale on which the degradation mechanism responds to the substrate, and t_0 can be interpreted as a lag between the start of the experiment and the time that substrate reaches the degradation mechanism. As already noted, the final value of percent degradation, D_0, is determined by the partition coefficient, f, and it is independent of the form of the rate equation. The basic nonlinearity of the model is reflected in the condition that $0 < f < 1$ (which is equivalent to requiring that $0 < D < 100\%$); the model becomes linear in the limiting cases $f = 0$ or $f = 1$.

In summary, the experimental findings presented here and the kinetic model which accounts for the data lead us to conclude that basal degradation of newly synthesized collagen is a posttranslational operation which occurs in a pathway that is kinetically distinct from the pathway leading to secretion. We emphasize, however, that this conclusion does not imply that basal degradation occurs in a subcellular compartment that is morphologically distinct from the sites of other post-translational processes. We are currently pursuing studies to determine the locus of basal degradation.

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Inactivation of D-(−)-β-Hydroxybutyrate Dehydrogenase by Modifiers of Carboxyl and Histidyl Groups†

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ABSTRACT: Data presented in this paper suggest that D-(−)-β-hydroxybutyrate dehydrogenase (BDH) purified from bovine heart mitochondria contains an essential carboxyl group and an essential histidyl residue at or near the active site. Lactate and malate dehydrogenases, which catalyze reactions analogous to that catalyzed by BDH, also contain an aspartyl and a histidyl residue at the active site [Birktoft, J. J., & Banaszak, L. J. (1983) *J. Biol. Chem. 258*, 472–482]. In addition, all three enzymes contain an essential arginyl residue, apparently concerned with electrostatic interaction with their respective carboxylic acid substrates, and promote ternary adduct formation involving the enzyme, NAD, and sulfite.

D-(−)-β-Hydroxybutyrate dehydrogenase (BDH,[1] EC 1.1.1.30) is a phospholipid-requiring enzyme that is associated with the mitochondrial inner membrane. BDH catalyzes with ordered bi bi kinetics the reversible oxidation of D-(−)-β-hydroxybutyrate to acetoacetate in the presence of NAD. The enzyme has been purified from bovine heart (Bock & Fleischer, 1975) and rat liver (Kebbaj et al., 1982) mitochondria. The bovine enzyme has a monomer M_r of 31 500, and the phospholipid-reconstituted holoenzyme has been reported to be tetrameric (McIntyre et al., 1983) or a mixture of aggregates (Yamaguchi & Hatefi, 1985). BDH contains essential arginyl (Phelps & Hatefi, 1981a; Kebbaj et al., 1982;

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[1] Abbreviations: BDH, LDH, and MDH, β-hydroxybutyrate, lactate, and malate dehydrogenases, respectively; DCCD, *N,N'*-dicyclohexylcarbodiimide; EDC, *N*-ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; EFA, ethoxyformic anhydride; *p*HMB, *p*-(hydroxymercuri)benzoate; SDS, sodium dodecyl sulfate; His, histidine; Asp, aspartic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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Fleer & Fleischer, 1983) and thiol (Sekuzu et al., 1963; Latruffe et al., 1980; Phelps & Hatefi, 1981b; Fleer et al., 1984) groups, apparently at or near the active site.

The reaction catalyzed by BDH is analogous to those catalyzed by lactate and malate dehydrogenases. They all involve the reversible oxidation of a short-chain hydroxy acid to a keto acid annd utilize NAD as cofactor. This similarity suggested to us earlier (Phelps & Hatefi, 1981a) that the catalytic mechanisms of these enzymes may involve common features. This paper shows that BDH appears to contain essential carboxyl and histidyl groups at the catalytic site. LDH and MDH also contain at the catalytic site an essential arginyl residue and a His-Asp pair in hydrogen-bonding proximity (Birktoft & Banaszak, 1983).

Materials and Methods

The sources of materials used were as follows: Sigma for sodium DL-β-hydroxybutyrate, lithium acetoacetate, snake venom phospholipase A2, EEDQ, EDC, EFA, *N*-ethylmaleimide, hydroxylamine monohydrochloride, 2-methylmalonate, *p*HMB, and dansyl chloride; Calbiochem for NADH, Hepes, dithiothreitol, and bovine serum albumin; Chemical Dynamics for NAD; Mallinckrodt for EDTA; Aldrich for DCCD; Research Products International for [^{14}C]DCCD; Associated Concentrates for asolectin; Schwartz/Mann for Tris.

Purification, Activation with Phospholipids, and Assay of BDH. BDH was purified from bovine heart submitochondrial particles according to Bock and Fleischer (1974). The purified enzyme was stored at −70 °C at a concentration of 1–2 mg/mL in a buffer containing 10 mM Hepes, 2 mM EDTA, 5 mM dithiothreitol, 0.4 M LiBr, and 20% (v/v) glycerol. Addition of glycerol protected the enzyme against inactivation by freeze–thawing and storage at −70 °C. The purified BDH had a specific activity of 50–60 μmol of NAD reduced·min^{-1}·(mg of protein)$^{-1}$ when it was reconstituted with optimal amounts of asolectin (65 μg of phospholipid phosphorus/mg of protein). The activity was twice as much when the apoenzyme was reconstituted with total mitochondrial phospholipids.

Aqueous dispersions of phospholipids were prepared by sonicating mitochondrial phospholipids or asolectin (30 mg/mL) in 20 mM Tris-HCl, pH 8.1, containing 1 mM EDTA, followed by removing the undispersed material by centrifugation for 30 min at 100000*g*. Reconstitution of the apoenzyme with phospholipids was carried out as follows. The apoenzyme (100 μL of 1 mg of protein/mL in 10 mM Hepes, 2 mM EDTA, 0.4 M LiBr, 5 mM dithiothreitol, and 20% glycerol, pH 7.0) was incubated with a predetermined optimal amount of the phospholipid suspension (6.5 μg of phosphorus) for 10–20 min at 37 °C. The activated enzyme was either used immediately or kept on ice before use, where it was stable for 5–6 h. For protein modification, the phospholipid-reconstituted enzyme (0.2 mL) was passed through a Sephadex G-50 column (1 mL) equilibrated in 20 mM potassium phosphate and 1 mM EDTA, pH 6.0, to remove dithiothreitol and other additives before addition of the modifying reagent.

Enzyme activity was assayed according to Bock and Fleischer (1974), except that the NAD concentration was 4 mM. Protein was estimated by the method of Peterson (1977) and phosphorus by the method of Ames (1966).

Treatment of BDH with [^{14}C]DCCD and Determination of Protein-Bound Radioactivity. Phospholipid-reconstituted BDH (0.5 mL of 1 mg/mL) was passed through a Sephadex G-50 column equilibrated in 20 mM potassium phosphate, pH 6.0, containing 1 mM EDTA. Where indicated, a similar batch of BDH was passed through Sephadex, the eluate (~2.0 mL) was treated with 10 μM *p*HMB and assayed at intervals until enzyme activity was completely inhibited. The material was then passed through a second Sephadex column as above to remove unreacted *p*HMB. The untreated and *p*HMB-treated samples were diluted with the same buffer to a protein concentration of 60 μg/mL and treated with [^{14}C]DCCD. At 10-min intervals, 10-μL aliquots were withdrawn and assayed for activity. The presence of 10 mM dithiothreitol in the assay medium reversed the inhibition due to *p*HMB but not that due to DCCD. At the same time intervals, 0.5-mL aliquots were removed and passed through two consecutive 3-mL Sephadex G-25 columns equilibrated in the phosphate–EDTA buffer to remove free DCCD. A portion of the eluate was denatured in 1% SDS and subjected to gel electrophoresis, and the remainder was used for protein determination. SDS–polyacrylamide gel electrophoresis was performed according to Laemmli (1970), with a 10% gel containing 4 M urea and 1 mM EDTA. The gels were stained with Coomassie blue and destained, the protein bands were cut and digested, and their radioactivity was determined as described elsewhere (Yamaguchi & Hatefi, 1985).

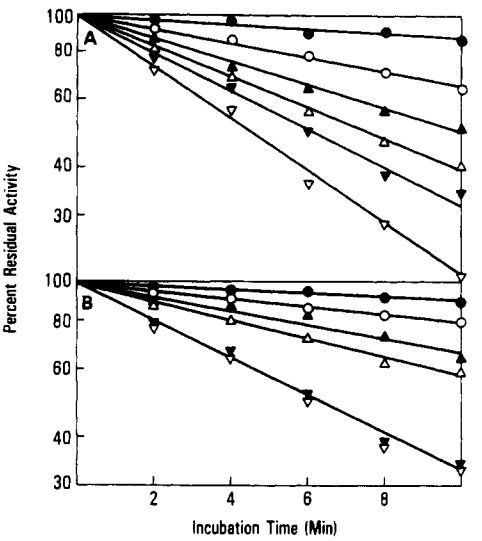

FIGURE 1: Semilogarithmic plots of the inhibition time course of BDH by DCCD at various inhibitor concentrations (A) or in the presence of substrates (B). Phospholipid-reconstituted BDH at 22 μg/mL in 20 mM potassium phosphate, pH 6.0, containing 0.5 mM EDTA was treated at 22 °C in (A) with 0 (●), 10 (○), 20 (▲), 30 (△), 40 (▼), and 60 (▽) μM DCCD and in (B) with 0 (●) or 40 (○, ▲, △, ▼, ▽) μM DCCD. Where indicated in (B), the following were added to BDH 5 min prior to the addition of DCCD: none (▽), 2 mM acetoacetate or 1 mM 2-methylmalonate (▼), 10 mM NAD (△), 1 mM NADH (▲), and 10 mM NAD plus 1 mM 2-methylmalonate (○). At 2-min intervals, aliquots of the enzyme were withdrawn and assayed for β-hydroxybutyrate dehydrogenase activity. The concentration of ethanol added as DCCD solvent to the enzyme incubation mixture was always less than 1%.

Results

Inhibition of BDH by DCCD, EEDQ, and EDC. Figure 1A shows a semilogarithmic plot of the inhibition time course of BDH treated with several different concentrations of DCCD. Similar results were obtained when the enzyme was incubated with EEDQ or EDC (data not shown). The second-order rate constants calculated for EEDQ, DCCD, and EDC were respectively 5.17×10^3, 2.2×10^3 and 0.11×10^3 M^{-1}·min^{-1}. Plots of the logarithms of inhibition rate constants vs. the logarithms of the corresponding EEDQ, DCCD, or EDC concentration showed in each case a straight line with a slope of unity (data now shown), suggesting that inhibition of BDH by each inhibitor is associated with modification of

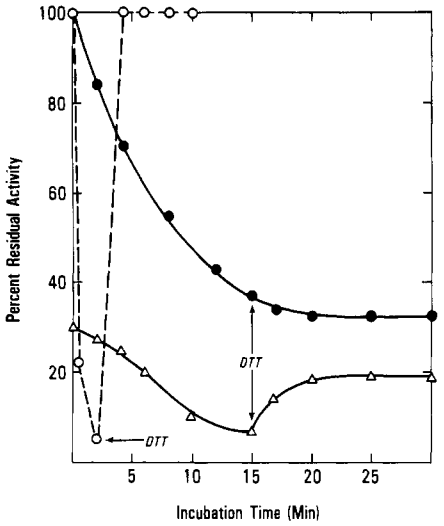

FIGURE 2: Effect of prior thiol modification on inhibition of BDH by DCCD. BDH at the same concentration and in the same buffer as in Figure 1 was incubated with 2 μM *p*HMB (○), 40 μM DCCD (●), or 1 μM *p*HMB followed by 40 μM DCCD (△). At the indicated time intervals, aliquots were withdrawn and assayed for activity in the absence of dithiothreitol in the assay mixture. Where shown by arrow, 10 mM dithiothreitol (DTT) was added to the incubation mixtures, and activity assays were continued as before.

a single residue per active unit of the enzyme (Levy et al., 1963). High BDH concentrations (e.g., >100 μg/mL) interfered with inhibition by DCCD when the latter was added at concentrations less than 50 μM. This problem was avoided when the ratio of DCCD to BDH was maintained at a level of at least 0.7–0.8 nmol of DCCD/μg of BDH up to a BDH concentration of 100 μg/mL. When EEDQ was the inhibitor, higher BDH/EEDQ ratios could be used without difficulty. Nevertheless, in all cases the BDH concentration used for modification by the above reagents was kept below 100 μg/mL. As shown in Figure 1B, NADH or NAD protected BDH against inhibition by DCCD. Consistent with the ordered mechanism of BDH, addition of the carboxylic acid substrates or the competitive inhibitor 2-methylmalonate in the absence of NAD(H) offered no protection, but addition of NAD(H) plus 2-methylmalonate resulted in nearly complete protection. Similar substrate protection effects were observed when the inhibitor was EEDQ or EDC (data not shown), suggesting that the essential residue modified might be at or near the enzyme active site.

Although enzyme inhibition by DCCD, EEDQ, and EDC is usually indicative of carboxyl group modification, other protein residues such as cysteine and tyrosine can also be modified by these reagents. Carbodiimide adducts of tyrosine are known to be reversible by nucleophiles such as hydroxylamine (Carraway & Koshland, 1972). However, when a sample of BDH that had been ~50% inhibited by DCCD was treated with hydroxylamine and assayed for activity after removal of hydroxylamine by filtration through a Sephadex column, there was no reversal of inhibition. The possibility that the essential thiol of BDH might be the target for DCCD or EEDQ was checked in a double-inhibition experiment. As seen in Figure 2, the inhibition of BDH by *p*HMB [also by a number of other thiol modifiers, including diamide (Phelps & Hatefi, 1981a,b), phenylarsene oxide, Cd^{2+}, cupryl-*o*-phenanthroline], but not by DCCD, is readily and completely reversed by the addition of dithiothreitol. However, a preparation of BDH whose essential thiol had been protected by *p*HMB (or phenylarsene oxide, data not shown) could be independently inhibited by subsequent treatment with DCCD.

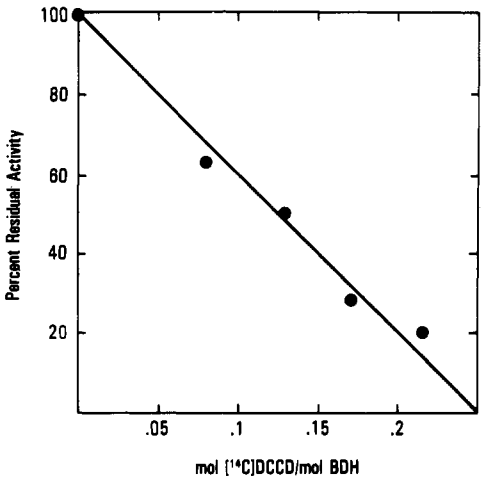

FIGURE 3: Correlation between activity and [^{14}C]DCCD incorporation into *p*HMB-treated BDH. Phospholipid-activated BDH was treated with *p*HMB as described under Materials and Methods. The *p*HMB-inactivated enzyme (60 μg/mL) was treated with 29 μM [^{14}C]DCCD (26 μCi/μmol). The inhibition of BDH by [^{14}C]DCCD and incorporation of radioactivity into the protein were determined as described under Materials and Methods.

As seen in Figure 2, addition of dithiothreitol to the enzyme treated with *p*HMB and DCCD did not cause complete reversal of inhibition but reversed only that fraction of the activity that had been inhibited by *p*HMB but not by DCCD. Similar results were obtained when, instead of DCCD, EEDQ was used as the second inhibitor (data not shown).

When phospholipid-reconstituted BDH was inhibited with [^{14}C]DCCD or with EEDQ in the presence of [^{3}H]aniline (Phelps & Hatefi, 1984; Laikind et al., 1985), there was no incorporation of radioactivity into the protein. This was not due to DCCD- or EEDQ-promoted enzyme dimerization, as evidenced from SDS–polyacrylamide gel electrophoresis of the inhibited enzyme, nor due to displacement of [^{14}C]DCCD or EEDQ by nucleophilic attack at the activated carboxyls by the phosphatidylethanolamine component of the added phospholipids. The completely inhibited enzyme was freed of phospholipids by multiple extractions with chloroform/methanol (2:1) (Wessel & Flugge, 1984) and assayed for protein-bound phosphorus (Ames, 1966). The analysis showed <0.07 mol of phosphorus/mol of BDH. This point was further supported by the fact that the phospholipid-free apoenzyme was also inhibited by [^{14}C]DCCD without incorporating radioactivity. Another possibility was displacement of DCCD or EEDQ from the activated carboxyl group by a nearby nucleophilic residue of the enzyme itself. Possible candidates were amino, carboxyl, hydroxyl, and thiol groups. BDH was not inhibited by pyridoxal phosphate or fluorescamine (dansyl chloride inhibits by modifying the essential thiol), thus suggesting the possible absence of an essential lysine. However, when the essential thiol, which, like the essential carboxyl appears to be located at the active site, was modified by *p*HMB or *N*-ethylmaleimide and then the enzyme was reacted with [^{14}C]DCCD, some radioactivity was incorporated into BDH. The level of [^{14}C]DCCD incorporation at 100% extrapolated inhibition corresponded to about 0.25 mol/mol of enzyme (Figure 3). Since radiation-inactivation studies have suggested that BDH in solution is tetrameric (McIntyre et al., 1983), the value of 0.25 mol of DCCD/mol of BDH could be considered to suggest that modification of one monomer by DCCD inhibits the activity of the tetramer. However, this interpretation does not agree with data regarding inhibitory modification of BDH by *N*-ethylmaleimide (McIntyre et al., 1984) and EFA (see below).

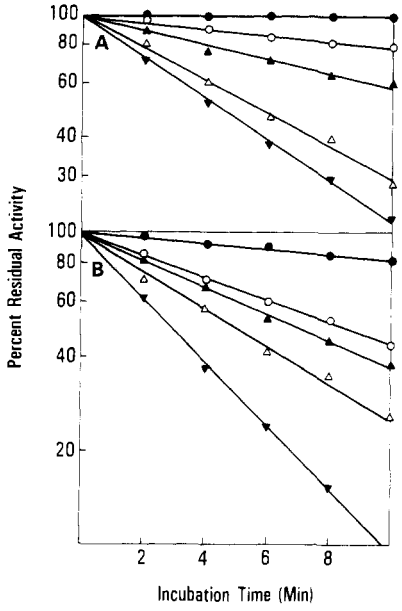

FIGURE 4: Semilogarithmic plots of the inhibition time course of phospholipid-reconstituted BDH (A) and the apoenzyme (B) by EFA at various concentrations. (A) Phospholipid-reconstituted BDH at 20 μg/mL in 0.1 M potassium phosphate, pH 6.0, containing 1 mM EDTA was incubated at 22 °C with 0 (●), 0.5 (○), 1.0 (▲), 2.0 (△), and 2.5 (▼) mM EFA, which had been freshly diluted into ethanol. Solvent concentration in the incubation mixtures was ≤2.5%. Samples were withdrawn at the indicated intervals and assayed for activity. (B) Apo-BDH was dissolved at 30 μg/mL in 20 mM potassium phosphate, pH 6.0, containing 1 mM EDTA, incubated for 5 min at 22 °C, and then treated with 0 (●), 0.05 (○), 0.075 (▲), 0.1 (△), and 0.2 (▼) mM EFA. At 2-min intervals, 0.1-mL aliquots were withdrawn and mixed with 0.8 mL of an assay mixture containing 10 mM potassium phosphate, pH 7.4, 0.5 mM EDTA, 0.5 mg of bovine serum albumn, 0.4% ethanol, 3 mM dithiothreitol, 4 mM NAD, and asolectin (200 ng of lipid phosphorus). After 10-min incubation at 37 °C, 0.1 mL of 0.2 M DL-β-hydroxybutyrate was added and NAD reduction followed spectrophotometrically at 340 nm.

The finding that modification of the essential thiol by *p*HMB or *N*-ethylmaleimide resulted in fractional incorporation of [^{14}C]DCCD into BDH suggested that [^{14}C]DCCD inhibition of the native enzyme might result in thio ester formation and release of [^{14}C]dicyclohexylurea. However, attempts at detecting thio ester formation in the DCCD-modified enzyme (Lipmann & Tuttle, 1945) were unsuccessful, and as mentioned earlier, treatment of the DCCD-inhibited enzyme with hydroxylamine did not result in reversal of inhibition. We think that the reason pretreatment of BDH with *p*HMB or *N*-ethylmaleimide leads to fractional incorporation of radioactivity when the thiol-modified enzyme is subsequently treated with [^{14}C]DCCD might be as follows. The essential carboxyl and the essential thiol are closely located. Modification of the thiol by *p*HMB or *N*-ethylmaleimide restricts the easy approach of a nucleophile to the essential carboxyl. Hence, when the latter reacts with DCCD to form the initial *O*-acyldicyclohexylisourea adduct, this unstable adduct is not as easily attacked by the nearby nucleophile as it is in the enzyme not containing a bulky group attached to the essential thiol. Consequently, a fraction of the isourea adduct rearranges to the *N*-substituted [^{14}C]dicyclohexylurea, which is stable.

Inhibition of BDH by EFA. Figure 4 shows semilogarithmic plots for the inhibition time course of phospholipid-reconstituted BDH (A) and the apoenzyme (B) in the presence of several different concentrations of EFA at pH 6.0. The second-order inhibition rate constants calculated from these data were 0.11 × 10^3 and 1.05 × 10^3 M^{-1}·min^{-1} for the holoenzyme and the apoenzyme, respectively. Spectrophotometric studies of BDH in the presence of EFA showed a time-dependent absorbance increase with a peak at about 234.5 nm, indicative of (ethoxyformyl)histidine. There was no trough at 280 nm, suggesting that treatment of BDH with EFA did not result in modification of tyrosyl residues (Burstein et al., 1974). Analysis of the spectrophotometric data for the number of histidine residues modified by EFA at different stages of BDH inhibition suggested that 100% extrapolated inhibition corresponded to modification by EFA of about 1.2 histidines per mole of BDH (Figure 5). This result agreed with estimation of the reaction order from the data of Figure 4A. When the logarithms of the inhibition rate constants were plotted against the logarithms of the corresponding EFA concentrations, a straight line was obtained with a slope of 1.09, suggesting the inhibition of BDH was the result of interaction of 1 mol of EFA with one active unit of the enzyme (Levy et al., 1963).

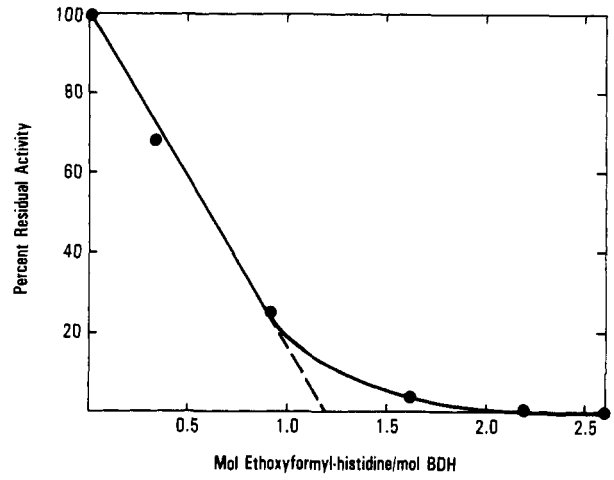

FIGURE 5: Correlation between inhibition of BDH and the number of histidyl residues modified by EFA. Apo-BDH (2 mL) was dialyzed for 4 h at 4 °C against 1 L of argon-saturated 0.1 M potassium phosphate, pH 6.0, containing 1 mM EDTA, 0.4 M LiBr, 0.5 mM dithiothreitol, and 10% (v/v) glycerol. The dialyzed enzyme (0.5 mg/mL) was placed in equal amounts in 1-mL sample and reference cuvettes. Freshly diluted EFA (0.1 M) was added to the sample in 1-μL aliquots and an equal volume of ethanol to the reference. The mixtures were incubated for 5 min at 22 °C, and their difference spectra were recorded from 330 to 230 nm by an Aminco DW-2a spectrophotometer. At the same time, 5-μL aliquots were withdrawn from each cuvette and reconstituted with asolectin as described in Figure 4B and their activities determined. (Ethoxyformyl)histidine formation was calculated from the difference spectra, with an extinction coefficient of 3200 M^{-1} cm^{-1} at 240 nm (Miles, 1977).

In addition to histidyl residues, EFA can modify tyrosyl, lysyl, cysteinyl, and arginyl residues. The spectrophotometric results discussed above eliminated tyrosyl modification, and the ineffectiveness of pyridoxal phosphate and fluorescamine as inhibitors suggested that BDH may not contain essential lysines. Also, BDH modification at the essential arginyl is prevented by the dicarboxylic acid substrates (in the presence of NAD or NADH) but not by the nucleotide cofactors alone, whereas NADH alone offered considerable protection to BDH against inhibition by EFA (see below). In addition, plots of the EFA inhibition rate constants of apo- and holo-BDH vs. pH in the pH range 5.5–8.5 suggested a pK_a close to pH 7.0 (data not shown), which agreed more with possible modification of an essential histidyl rather than a lysyl or an arginyl residue. The possibility of the essential thiol as the EFA target was also eliminated by a double-inhibition experiment. The progress of inhibition of BDH by 1 mM EFA was followed with time, as shown in Figure 6. In a parallel experiment,

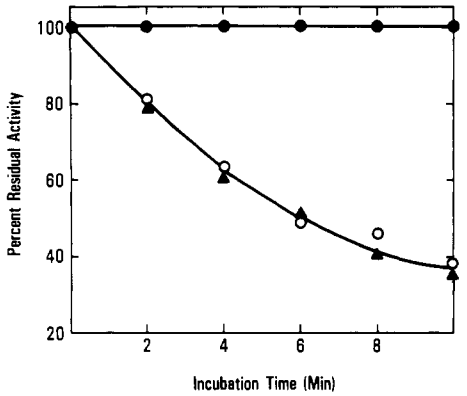

FIGURE 6: Effect of prior thiol modification on inhibition of BDH by EFA. Phospholipid-reconstituted BDH at 25 μg/mL in 20 mM potassium phosphate, pH 6.0, containing 1 mM EDTA was treated with 1 mM EFA (▲) or with 5 μM *p*HMB followed by 1 mM EFA (○). The mixtures and the untreated control (●) were incubated at 22 °C and sampled at 2-min intervals for activity assay in the presence of 10 mM dithiothreitol.

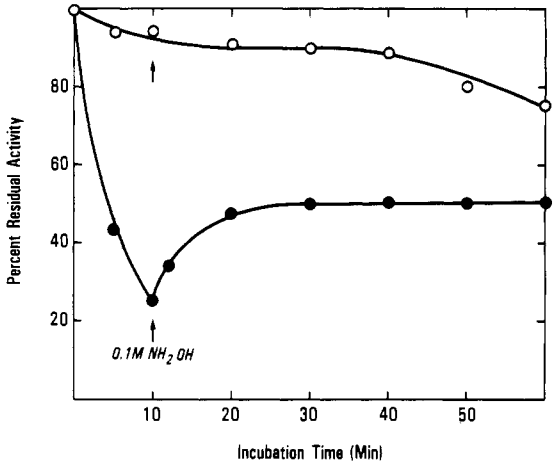

FIGURE 7: Reactivation of EFA-inhibited BDH by hydroxylamine. Apo-BDH at 30 μg/mL in 20 mM potassium phosphate, pH 6.0, containing 1 mM EDTA was treated with 0.1 mM EFA (●) or an equal volume of ethanol (○) and incubated at 22 °C. At the indicated intervals, 0.1-mL aliquots were removed, reconstituted with asolectin as described in Figure 4B, and assayed for activity. At the time shown by arrows, hydroxylamine (pH 7.0) was added to the EFA-treated and control incubation mixtures to a final concentration of 0.1 M. Samples were withdrawn at the indicated intervals, reconstituted with asolectin as before, and assayed for activity.

BDH was first inhibited completely by treatment with *p*HMB, then 1 mM EFA was added, and the progress of inhibition by EFA was followed as before. In both cases, the enzyme was assayed in the presence of 10 mM dithiothreitol, which reversed the inhibition due to *p*HMB, thus allowing the inhibition due to EFA to be observed in the second experiment. As seen in Figure 6, inhibition by EFA of native and *p*HMB-treated BDH followed the same course, indicating that the target for EFA was not the essential thiol of the enzyme. Finally, the EFA inhibition of BDH could be partially reversed by treating the inhibited enzyme with hydroxylamine (Figure 7). The reversal by hydroxylamine is characteristic of EFA modification of histidyl and tyrosyl residues (Burstein et al., 1974; Miles, 1977). Modification by EFA of other amino acid residues is not reversed by hydroxylamine. The observation that hydroxylamine reversal of the EFA inhibition was only partial is not unusual and has been reported for many enzymes containing an EFA-modifiable essential histidine (Miles, 1977; Saluja & McFadden, 1982; Daron & Aull, 1982; Carillo & Vallejos, 1983; Hennecke & Plapp, 1983).

Table I: Effect of Substrates on Inhibition of BDH by EFA[a]

additions	$k - k_0$ (min^{-1})
none	0.059
NAD	0.060
NADH	0.014
β-hydroxybutyrate	0.089
acetoacetate	0.061
2-methylmalonate	0.060
NAD + β-hydroxybutyrate	0.040
NAD + acetoacetate	0.062
NAD + 2-methylmalonate	0.00
NADH + β-hydroxybutyrate	0.020
NADH + acetoacetate	0.040
NADH + 2-methylmalonate	0.00

[a] Asolectin-reconstituted BDH at 25 μg/mL in 50 mM potassium phosphate, pH 6.0, containing 1 mM EDTA was treated with 1 mM EFA at 22 °C. Aliquots were withdrawn at 2-min intervals and assayed for β-hydroxybutyrate dehydrogenase activity. Where indicated, BDH was incubated with the following ligands for 5 min at 22 °C before addition of EFA: 10 mM NAD, 1 mM NADH, 20 mM DL-β-hydroxybutyrate, 2 mM acetoacetate, and 1 mM 2-methylmalonate. In all preincubation mixtures, the salt concentration was adjusted to 50 mM by addition of appropriate amounts of NaCl. k and k_0 are respectively the pseudo-first-order inhibition rate constants in the presence and absence of EFA.

BDH was protected against EFA inhibition by NADH ± β-hydroxybutyrate (Table I). NAD or the carboxylic acid substrates alone and NAD plus acetoacetate showed no protection. However, NAD or NADH plus 2-methylmalonate at the concentrations shown in Table I offered complete protection. These and the above results are consistent with the possible presence of an essential histidyl residue at or near the active site of BDH.

Discussion

It has been shown that BDH purified from bovine heart mitochondria is inhibited by treatment with the carboxyl group modifiers EEDQ, DCCD, or EDC or by treatment with the histidyl residue modifier EFA. The reaction order for BDH inhibition by each reagent was unity. The enzyme was protected against EEDQ, DCCD, or EDC inhibition by NAD(H) + 2-methylmalonate > NADH > NAD and against EFA inhibition by NAD(H) + 2-methylmalonate > NADH > NAD + β-hydroxybutyrate or NADH + acetoacetate. The possible involvement of the essential thiol of BDH as a target for the above reagents was eliminated by double-inhibition experiments, which showed that BDH could still be inhibited by these reagents when the essential thiol was reversibly protected. Other residues that were eliminated or appeared unlikely were tyrosyl as a target for DCCD and tyrosyl, lysyl, and arginyl as targets for EFA. Histidine as a target for EFA appeared to be a strong possibility, because (i) the formation of 1 mol of (ethoxyformyl)histidine/mol of BDH was shown spectrophotometrically to correlate with enzyme inhibition, (ii) the pK_a of the essential residue modified by EFA appeared to be close to pH 7.0, and (iii) the EFA inhibition was partially reversed by hydroxylamine. The above results suggested, therefore, that BDH contains an essential carboxyl group and an essential histidyl residue at or near the active site.

When BDH was treated with [^{14}C]DCCD or with EEDQ in the presence of [^{3}H]aniline, the enzyme was inhibited without incorporation of radioactivity, indicating elimination of [^{14}C]DCCD or EEDQ. Appropriate experiments showed that reagent elimination was not due to enzyme dimerization by cross-linking nor due to amide formation between the activated carboxyls of BDH and phosphatidylethanolamine of the added phospholipids. The possibility that the activated essential carboxyl might be reacting with the active site es-

sential thiol to form a thio ester linkage was also considered. Although no thio ester could be detected in the DCCD-inhibited enzyme, the possibility of thio ester formation could not be ruled out because of the following observation. When the essential thiol of BDH was protected by *p*HMB (or irreversibly modified by *N*-ethylmaleimide), subsequent treatment with [^{14}C]DCCD resulted in radioactivity incorporation up to 0.25 mol of [^{14}C]DCCD/mol of BDH at 100% extrapolated enzyme inhibition (Figure 3). However, the fact that an enzyme preparation that had been completely inhibited by *p*HMB or *N*-ethylmaleimide did not incorporate more [^{14}C]DCCD in several experiments, involving different high ratios of [^{14}C]DCCD to protein and prolonged (up to 16 h) incubation times, suggested another possibility more consistent with the results. Suppose the nucleophile attacking the activated carboxyl is different from the essential thiol, but the thiol is located near the essential carboxyl, and its modification by *p*HMB or *N*-ethylmaleimide restricts the easy approach of the nucleophile to the activated carboxyl. Under these conditions, one might expect a fraction of the *O*-acyl[^{14}C]dicyclohexylisourea formed initially to rearrange slowly to the stable *N*-substituted urea derivative and the remainder to undergo nucleophilic attack and loss of [^{14}C]dicyclohexylurea.

The possible presence of an essential carboxyl group and an essential histidyl residue at the active site of BDH is especially interesting in view of the fact that LDH and MDH, which catalyze analogous reactions, have been shown to contain a His-Asp pair at the active site (Birktoft & Banaszak, 1983). The His-Asp pair of LDH and MDH is thought to participate in the catalytic process via a charge-relay system involving hydrogen bonding between one imidazole nitrogen of His and the free carboxyl of Asp, as well as between the second imidazole nitrogen of His and the substrate hydroxyl/keto group, thus making the C-2 of lactate/pyruvate or malate/oxaloacetate susceptible to oxidation or reduction by NAD or NADH, respectively. A similar mechanism can be conceived for BDH (Phelps & Hatefi, 1981a). Indeed, as pointed out elsewhere (Phelps & Hatefi, 1981a), the pH optima for β-hydroxybutyrate oxidation (pH $\sim$8.0) and acetoacetate reduction (pH $\sim$7.0) agree with the possible participation in catalysis of the unprotonated essential histidine as a proton acceptor during oxidation of β-hydroxybutyrate and the protonated essential histidine as a proton donor during reduction of acetoacetate. Another feature common to LDH, MDH, and BDH is that they all contain an essential arginyl, apparently involved in electrostatic interaction with the substrate carboxyl (Phelps & Hatefi, 1981a; Birktoft & Banaszack, 1983). In addition, all three enzymes are capable of promoting ternary complex formation involving the enzyme, NAD, and sulfite (Parker et al., 1978; Phelps & Hatefi, 1981b; Fritzsche et al., 1983).

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Registry No. BDH, 9028-38-0; EEDQ, 16357-59-8; DCCD, 538-75-0; EDC, 1892-57-5; NADH, 58-68-4; NAD, 53-84-9; EFA, 1609-47-8; $HO_2CCH(Me)CO_2H$, 516-05-2; L-His, 71-00-1; L-Arg, 74-79-3.

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